Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation-flocculation pretreatment

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Abstract

Hospital wastewaters are a main source of pharmaceutical active compounds, which are usually highly recalcitrant and can accumulate in surface and groundwater bodies. Fungal treatments can remove these contaminants prior to discharge, but real wastewater poses a problem to fungal survival due to

bacterial competition. This study successfully treated real non-spiked, non-sterile wastewater in a continuous fungal fluidized bed bioreactor coupled to a coagulation-flocculation pretreatment for 56 days. A control bioreactor without the fungus was also operated and the results were compared. A denaturing gradient gel electrophoresis (DGGE) and sequencing approach was used to study the microbial community arisen in both reactors and as a result some bacterial degraders are proposed. The fungal operation successfully removed analgesics and anti-inflammatories, and even the most recalcitrant pharmaceutical families such as antibiotics and psychiatric drugs.

Keywords: fungal bioreactor, pharmaceutical active compounds, continuous treatment, non-sterile, hospital wastewater, pretreatment

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1. Introduction

Pharmaceutical active compounds (PhACs) have been found in water bodies at significant concentrations (Gros et al., 2012). The primary source of these contaminants in the environment is known to be through wastewater treatment plant (WWTP) effluents, not designed to remove these emerging pollutants (Deblonde and Hartemann, 2013). PhACs are found at especially high concentrations (up to $\mu g \cdot L^{-1}$ and $mg \cdot L^{-1}$) in hospital wastewater (HWW), which is typically discharged untreated into the sewer network. In consequence, the on-site treatment of these hospital effluents prior to discharge has arisen as a promising possibility (Verlicchi et al., 2012). These recalcitrant compounds

would be total or partially degraded and transformed into more degradable compounds for further downstream treatment.

White-rot fungi (WRF) have demonstrated the capability to degrade several PhACs and consequently, a fungal approach to treat on-site hospital effluents emerges as an attractive perspective. First studies on fungal treatment performance concerning pharmaceutical removal were carried out in sterile conditions and with single-spiked pollutants (Marco-Urrea et al., 2009, 2010; Jelic et al., 2012). Studies in non-sterile more complex matrices are scarcer but have demonstrated the ability of fungi to transform and/or remove PhACs from non-sterile HWW (Cruz-Morató et al., 2013). One of the drawbacks of the technology in non-sterile conditions is the difficulty in maintaining the fungal activity for a long period of time since bacteria exert competitive pressure in fungal survival. The implementation of a coagulation-floculation step before the fungal treatment of spiked HWW reduced the microbial load of the influent thus allowing the maintenance of fungal activity for 28 days (Mir-Tutusaus et al., 2016). Furthermore, a partial biomass renovation, previously described by Blánquez et al. (2006), could extend the treatment by overcoming the biomass aging process. This approach has been implemented and is discussed in the present manuscript. To approach a real application, a non-spiked matrix is preferred.

Additionally, despite some studies have investigated the bacterial and fungal communities in fungal bioreactors treating wastewater (Badia-Fabregat et al., 2015), it still remains unclear which microorganisms are responsible for the PhACs elimination. The assessment of microbial assemblage would enhance

the knowledge about this type of systems and help in the design of future treatments.

This study provides the validation of previous work in spiked HWW (Mir-Tutusaus et al., 2016), while approaching real application. The main focus of the manuscript has been the discussion of PhACs removal and its relation to microbial community evolution. Moreover, a long operation of this kind of reactors in non-sterile HWW has never been achieved before and it would signify a promising step in the maturation of fungal technology in wastewater treatment. The objectives of the study are thus to test the ability of WRF *Trametes versicolor* to treat real non-sterile HWW after a coagulation-flocculation pretreatment for a long period of time, to evaluate the bacterial and fungal communities arisen during the treatment and to assess the removal efficiency for PhACs.

2. Materials and methods

2.1. Reagents, fungus and hospital wastewater

All the pharmaceutical and the corresponding isotopically labelled standards used in the analysis were of high purity grade (>90%) and they were purchased from Sigma–Aldrich (Steinheim, Germany), US Pharmacopeia USP (MD, USA), Europea Pharmacopoeia EP (Strasbourg, France), Toronto Research Chemicals TRC (Ontario, Canada) and CDN isotopes (Quebec, Canada). Individual as well as isotopically labelled standard solutions were prepared according to Gros et al. (2012).Thiamine hydrochloride was acquired from

Merck (Barcelona, Spain), peptone and yeast extract from Scharlau (Barcelona, Spain) and glucose, ammonium chloride and other chemicals were purchased from Sigma-Aldrich (Barcelona, Spain). All other chemicals used were of analytical grade.

T. versicolor (ATCC#42530) was maintained on 2% malt agar slants at 25°C until use. Subcultures were routinely made. A mycelial suspension of *T. versicolor* was obtained as previously described by Blanquez et al (2004).

The HWW was collected directly from the sewer manifold of Sant Joan de Déu Hospital (Barcelona, Spain). Fresh samples were collected in two occasions prior to every experiment and stored at 4°C. The characteristics of the wastewaters are summarized in Table 1.

2.2. Medium and pellet formation

Fungal pellets were obtained as previously described (Mir-Tutusaus et al., 2016). The defined medium contained per liter: glucose 10 g, macronutrients 100 mL, micronutrients 10 mL, NH_4Cl 2.1 g and thiamine 10 mg (Borràs et al., 2008). The pH was controlled at 4.5 by adding HCl 1M or NaOH 1M and the saturation percentage of dissolved O_2 was measured to ensure proper aeration. Fluidized conditions in the reactors were maintained by using 1s air pulse every 4s. The aeration rate was 0.8 $L \cdot min^{-1}$ and the temperature was maintained at 25°C.

2.3. HWW treatment

Wastewater was pretreated with a coagulation-flocculation process. Coagulant HyflocAC50 and flocculant HimolocDR3000 were kindly provided by Derypol,

S.A. (Barcelona, Spain). The pretreatment involved 2 min of coagulation at 200 rpm, 15 min of flocculation at 20 rpm and 30 min of settling. HWW1 and HWW2 were pretreated with 95 mg·L-1 and 190 mg·$L^{-1}$of coagulant and 10 mg·L^{-1} and 20 mg·L^{-1} of flocculant, respectively.

After the pellet growth, the medium was withdrawn and the bioreactor was filled with the pretreated HWW. Two bioreactors were run in parallel, one inoculated with *T. versicolor* (RA) and one uninoculated control (RB), both operating continuously with a hydraulic residence time (HRT) of 3 days. HWW1 was used for the startup and during the first 29 days of operation of both RA (inoculated with *T. versicolor*) and RB (uninoculated control) bioreactors, whereas HWW2 was used for the following 27 days in both reactors. Nutrients for maintenance, glucose and NH_4Cl, were added with a molar C/N ratio of 7.5 at *T. versicolor* consumption rate to both reactors (1200 mg glucose·$gDCW^{-1}$·d^{-1}). A partial biomass renovation strategy was carried out in the fungal bioreactor, as described by Blánquez et al. (2006), with 1/3 of biomass renovated every 7 days which produced a cellular retention time (CRT) of 21 d.

2.4. Analysis of pharmaceuticals

The analytical procedure performed is based on Gros et al. (2012). Briefly, the samples were filtered through 0.45 µm glass fibber filters. Then, 25 mL of sample for raw HWW and 50 mL for treated wastewater were pre-concentrated by SPE (Solid Phase Extraction) using Oasis HLB (3cc, 60 mg) cartridges (Waters Corp. Mildford, MA, USA), which were previously conditioned with 5 mL of methanol and 5 mL of HPLC grade water. Elution was done with 6 mL of pure methanol. The extracts were evaporated under nitrogen stream and

reconstituted with 1 mL of methanol-water (10:90 v/v). 10 µL of internal standards mix at 1 ng·µL^{-1} in methanol were added in the extracts for internal standard calibration. Chromatographic separation was carried out with an Ultra-Performance liquid chromatography (UPLC) system (Waters Corp. Mildford, MA, USA), equipped with an Acquity HSS T3 column (50 mm x 2.1 mm i.d. 1.7 µm particle size) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7µm particle size) for the ones analyzed under negative electrospray ionization (NI), both from Waters Corporation. The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. Two multiple reaction monitoring (MRM) transitions per compound were recorded by using the Scheduled MRM^{TM} algorithm and the data were acquired and processed using Analyst 2.1 software.

2.5. Microbial Community Analysis

Liquid samples from each reactor were filtered through 0.22 µm GV Durapore® membrane filters (Merck Millipore, USA) and filters were stored at -80ºC. Sampled pellets were centrifuged at 14.000 rpm and liquid fraction was discarded before cold-storage at -80ºC. Total DNA extraction was conducted using the PowerWater® and PowerSoil® DNA Isolation Kits (MoBio Laboratories, USA) for filters and pellets, respectively. For bacterial analyses, a 550 bp DNA fragment in the 16S region of the small subunit ribosomal RNA gene was amplified using the primer set 341f/907r (Muyzer et al., 1993) with a GC clamp added at the 5' end of primer 341f. Final concentrations of the PCR reactions consisted of 1x PCR buffer, 2 mM of $MgCl_2$, 200 µM of each

deoxynucleoside triphosphate, 500 nM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen, ThermoFisher Scientific, USA). Amplification protocol consisted of: 94ºC for 5 min; 20 cycles of 94ºC for 1 min, 65ºC for 1 min (-0.5ºC/cycle), 72ºC for 3 min; 15 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 3 min; and a single final extension of 72ºC for 7 min. Fungal DNA was amplified using a nested approach over a ~400 bp fragment from the internal transcribed spacer (ITS) of fungal ribosomal RNA gene. The primer sets used were EF4/ITS4 and ITS1f-GC/ITS2 (Gardes and Bruns, 1993; White et al., 1990) for the first and second round of amplification, respectively. The GC clamp was added at the 5' side of primer ITS1f and PCR reactions had the same final concentrations except for MgCl2 (1.5 mM). PCR program for fungi was identical for both amplification rounds and consisted of: 95ºC for 5 min; 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s; and a single final extension 72 ºC for 5 min.

Denaturing gradient gel electrophoresis (DGGE) was performed using the Dcode Universal Mutation Detection System (Bio-Rad, Spain). 900 ng of DNA from PCR products were loaded onto 6% (w/v) polyacrylamide gels (acrylamide/bis solution 37.5:1) containing linear chemical gradients 30-70% denaturant for bacteria and 15-55% denaturant for fungi. 100% denaturing solution contained 7 M urea and 40% (v/v) deionized formamide. Gels were run in 1X Tris acetate-EDTA (TAE) for 16 h at 75 V and 60ºC, stained with 1 µg/mL ethidium bromide solution for 25 min, washed with deionized water 25 min and photographed with Universal Hood II (Bio-Rad, Spain). DGGE images were analysed using InfoQuest™ FP software. Dice's coefficient and unweighted pair group method with arithmetic averages (UPGMA) were employed for the

clustering of DGGE gel profiles. Prominent bands from the DGGE were excised, re-amplified and then sequenced by Macrogen (South Korea). Obtained sequences were trimmed with FinchTV software and checked for chimeras using Mothur (Schloss et al., 2009). Each 16S rRNA sequence was assigned to its closest neighbor according to the Basic Local Alignment Search Tool (BLAST) results (Altschul et al., 1997).

2.6. *Vibrio fischeri* bioluminescence inhibition test (Microtox® test)

Microtox acute toxicity bioassay kit from Azur Environmental (Carlsbad, US) was used in toxicity tests. Briefly, the test is based on the diminution of bacterial bioluminescence after 5 and 15 min of exposure to dilutions of the samples (pH 7). Toxicity was expressed as toxicity units (TU).

2.7. Other analyses

Glucose concentration was measured per triplicate with a YSI 2700 SELECT enzymatic analyzer (Yellow Spring Instruments). Laccase activity was measured per triplicate using the method previously described (Mir-Tutusaus et al., 2016). The conductivity was determined by a CRISON MicroCM 2100 conductimeter, and the absorbance at 650nm was monitored by a UNICAM 8625 UV/VIS spectrometer. Chloride, sulfate, nitrate and phosphate anions were quantified by a Dionex ICS-2000 ionic chromatograph. The total suspended solids (TSS), dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) were determined according to APHA-AWWA-WEF (1995). The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analyzed by using commercial kits LCH303 and LCK114 or LCK314, respectively (Hach Lange, Germany).

2.8. Statistical analysis

As recommended by Bolks et al. (2014), a robust Regression on Order Statistics (ROS) approach was used for dealing with left-censored data, namely: below limit of detection (BLD) and below limit of quantification (BLQ) values. When ROS was not possible, values BLD and BLQ were considered to have a concentration half of the limit of detection and half of the limit of quantification, respectively(EPA, 2000). ROS analysis was performed using the R package NADA (Lee, 2013). Other calculations like summary statistics, pharmaceutical removals and one-factor analysis of variance (ANOVA) were performed with R: A language and environment for statistical computing (R Core Team, 2015). Differences were considered as significant at $p < 0.05$.

3. Results

The results of HWWs characterization (Table 1) show that the measured physicochemical parameters were in the same range as other HWW. Pharmaceuticals concentrations, presented in Table 2 ranged from $ng \cdot L^{-1}$ to few $\mu g \cdot L^{-1}$, results also in agreement with previous studies (Badia-Fabregat et al., 2015; Cruz-Morató et al., 2013). The pretreatment diminished the absorbance at 650 nm from 0.215 and 0.265 to values very close to zero and the COD from 633 and 1012 mg $O_2 \cdot L^{-1}$ to 215 and 300 mg $O_2 \cdot L^{-1}$, respectively. This reduction is in accordance with previous experiments (Mir-Tutusaus et al., 2016).

As stated before, in the case of RA 1/3 of biomass was purged and renovated with fresh pellets every 7 days as an approach to maintain a stable and active culture in the bioreactor. The control reactor RB was not inoculated. A fungal control reactor without biomass renovation was not operated, so the impact of

biomass replacement in length of operation will not be included in the RA/RB comparison. A complete study on biomass renovation can be found elsewhere (Blánquez et al., 2006) in sterile conditions. In addition, present results can be compared to those previously described in the treatment of HWW in non-sterile conditions without biomass renovation (Mir-Tutusaus et al., 2016). The fungal reactor (RA) underwent an incident where pH was sustained below 3 for several hours. pH is critical in fungal systems, as supported by Borràs et al. (2008), and this low pH period leaded to a substantial loss of pelleted morphology. This incident took place during the change of HWW, circumstance that hampered the interpretation of results. The reactor recovered pelleted morphology and laccase production through weekly biomass renovation but behaved differently apropos of PhACs removal. The main conclusions of the treatment could be drawn from the first 28 days of operation; however, understanding the second period and the recovery from a low pH period could expand the knowledge of the system and give insight towards real application, hence all the results are presented.

3.1. Monitoring of bioreactors

The lack of on-line monitoring variables usually difficults the evaluation of the bioreactor performance. In this operation laccase activity and glucose concentration were measured during the treatments (Figure 1). Laccase activity was not detected in the reactors during the first period of operation (HWW1); however, *T. versicolor* produced the enzyme because when biomass was removed from the reactors, rinsed and placed in stirred Erlenmeyers with defined medium (*ex situ* assay), laccase activity could be measured. *Ex situ* assays detected laccase activity in RA but not in RB (data not shown). During

the second period of operation (HWW2) laccase profile in RA was irregular with peaks of over 45 $U \cdot L^{-1}$ at Days 30, 45 and 50. Laccase activity in the uninoculated reactor remained insignificant throughout the treatment. *T. versicolor* remained active during the whole treatment. However, it could not be asserted whether HWW1 interfered with the assay, the production of laccase, or some compounds in HWW1 inactivated the laccase. In complex matrices, the purification of the laccase is required prior to the measurement of the activity (D'Annibale et al., 2006) and it should be taken into accout in future studies. As previously discussed (Mir-Tutusaus et al., 2016), laccase production was sign of *T. versicolor* activity, but its absence was not an indication of the fungus inactivity. In fact, as argued in the Discussion section, RA showed high removal capacity when laccase activity was very low.

Glucose was added at *T. versicolor* consumption rate thus glucose concentration remained at values close to zero throughout the treatment in the fungal reactor. In RB glucose accumulated during the first two weeks of operation until it was colonized by HWW-native microorganisms and remained insignificant from that point onwards. Total COD is presented in Table 3. RA did not significantly increase initial COD except around the day of the pH incident. This agreed with the observed loss of pelleted morphology, as free hyphae increased the COD load. Contrarily, RB consistently exhibited COD around 1200 $mg \cdot L^{-1}$. The profiles of TSS concentration are presented in Fig. 2. RA profile was mostly constant at around 120 $mg \cdot L^{-1}$, but the control RB did not achieve a steady state, exhibiting a much more irregular profile with peaks of over 2000 $mg \cdot L^{-1}$ at the end of the treatment. Neither COD nor TSS levels reached the European Union standard of 125 $mg \cdot L^{-1}$ and 35 $mg \cdot L^{-1}$,

respectively, in either reactor (EEC Council, 1991); but the objective was to remove PhACs. The system is, as stated before, an on-site treatment prior to discharge to the sewer network.

3.2. PhACs removal and toxicity assessment

46 out of the 81 PhACs analyzed were detected during the treatments (Table 2). In 35 and 34 compounds were detected in raw wastewater HWW1 and HWW2, respectively, whereas after the pretreatment only 34 and 32. The two pretreated wastewaters used as influent had different PhAC composition. Overall, HWW1 had higher concentrations of detected PhACs than HWW2. The most common families of PhACs detected were analgesics and anti-inflammatories, antibiotics and psychiatric drugs, as the hospital has an important psychiatric pavilion. Initial concentrations of individual pharmaceuticals can be found in Table 2. The analgesics and anti-inflammatories family contribute the most to the final concentration, especially due to the high concentrations of ibuprofen and acetaminophen. Psychiatric drugs, led by 2-hydroxycarbamazepine and 10,11-epoxycarbamazepine (both known metabolites of carbamazepine), are the second group in concentration. Lipid regulators family ranked 3rd followed by antibiotics family.

Excluding analgesics and anti-inflammatories, which is a family known to be easily degraded, the amount of compounds was 17.8 $\mu g \cdot L^{-1}$ and decreased to 4 ± 1 $\mu g \cdot L^{-1}$ and 9 ± 1 $\mu g \cdot L^{-1}$ in reactors RA and RB, respectively, corresponding to a removal of 78 ± 7% and 48 ± 4%. In HWW2, when RA was recovering from the pH incident, the removal percentages excluding analgesics and anti-inflammatories were around 30-35% for both reactors.

Time-course profile of concentrations of the different families during the treatments is presented in Fig. 3. Analgesics and anti-inflammatories were present in a high concentration, but were rapidly removed by both the inoculated and the uninoculated reactor with removal values of above 80%, as both bacteria and fungi are reported to remove these compounds (Langenhoff et al., 2013). Both reactors exhibited an increase in removal capacity from day 9 to day 27, around the time of the change in wastewater, which contained approximately half of the concentration of PhACs than HWW1. From that point onwards, both RA and RB behaved steadily with removal values of around 90%.

Antibiotics initial concentration was around 5000 $ng \cdot L^{-1}$. *T. versicolor* was able to remove 90% of its initial load but gradually lost removal capacity to values around 50%. RB did not significantly remove antibiotics; its concentrations remained equal or increased. Since the pH incident, RA did not recover removal capacity. RB continued to exhibit higher antibiotics concentration than the inlet when treating HWW2.

The presented psychiatric drugs family excludes carbamazepine (CBZ) and its transformation products (TP); carbamazepine case is discussed below because it is an especially recalcitrant compound not removed in conventional WWTPs and information on the TPs was available (Clara et al., 2004). *T. versicolor* was able to remove around 50% of the initial load of psychiatric drugs, although its initial removal was 86%. The uninoculated reactor did not remove this family and presented higher concentrations than the inlet during all the treatment.

The concentration profile of other pharmaceutical compounds is particularly different in the two wastewaters. The first 27 days showed nearly constant concentrations of around 900 $ng \cdot L^{-1}$ in the fungal reactor and around 2500 $ng \cdot L^{-1}$ in RB. This translates to removal values of circa 90% for RA and 70% for RB. During the second part of the treatment, both reactors behaved similarly with removals around 60%. None of the compounds detected had anti-fungal activity.

The case of carbamazepine and its transformation products 10,11-carabamazepine, 2-hydroxycarbamazepine and acridone is presented in Fig. 4. Carbamazepine is mainly metabolized in the liver, generating the analyzed transformation products, among other metabolites as well as several glucuronide conjugates (Kaiser et al., 2014). Both HWW1 and HWW2 contained similar concentrations of CBZ and its TPs, excluding 10,11-expoxyCBZ, with a concentration much more higher in HWW2 than in HWW1. The fungal bioreactor was able to remove from 50 – 80% of CBZ, around 50% of 10,11-epoxyCBZ and nearly 100% of 2-hydroxyCBZ, but the concentration of acridone increased. While recovering from the pH incident, RA behaved differently: it retained the 2-hydroxyCBZ removal capacity and the concentration of acridone increased, but it exhibited low removals of CBZ and 10,11-epoxyCBZ. RB was not able to remove CBZ from the wastewaters. It showed, nonetheless, good removal values of carbamazepine TPs but lost removal capacity from day 27 onwards. After day 42 RB regained its ability to remove 10,11-epoxyCBZ and small amounts of 2-hydroxyCBZ; concentration of acridone also increased.

Regarding the toxicity assessment, the fungal reactor maintained the toxicity at 0 TU during the whole treatment. Contrarily, the control bioreactor raised toxicity to 27 TU.

3.3. Evolution of bacterial and fungal populations

The evolution of fungal and bacterial populations was studied by DGGE analysis and DNA sequencing. Two DGGE gels (one for bacteria and one for fungi) were run with samples collected from the liquid matrix of RA (RA), from the pelleted biomass in RA (PRA) and from the liquid matrix of RB (RB). The results are presented in figures 5-6 and the DGGE profiles in supplementary material (Figure S1). Microbial communities in both reactors changed during the treatment and longer operations might be needed to achieve a steady state.

64 prominent bands from the fungal DGGE were excised and sequenced, obtaining 97% coverage of the phylotypes associated with the quantitative DGGE band matrix. Representative sequences were submitted to the GeneBank database under the accession numbers KX530041 to KX530058. While only two phyla (Ascomycota and Basidiomycota) were represented in the fungal sequences, these were composed by 7 different genera: *Candida*, *Isaria*, *Phialemoniopsis* and *Trichoderma* from phyla Ascomycota and *Asterotremella, Trametes* and *Tremella* from phyla Basidiomycota (supplementary material table S1).

At the genus level, *Trametes* was consolidated all along the operation in pellet samples (PRA). When pellets started losing shape, *T. versicolor* was also found in the supernatant samples (RA). *Tremella* and *Asterotremella* were dominant in RA reactor initially, until *Candida* was established in mid-late period after the pH

incident. Moreover in RB reactor *Asterotremella* and *Trichoderma* predominated before the change of WW, then a substitution of the former for *Phialemoniopsis* was observed from day 21 onwards.

In parallel, 74 prominent bands from the bacterial DGGE were excised and sequenced. In this case the community coverage also stood in 97%. Sequences from each phylotype were submitted to GeneBank under accession numbers KX523866 to KX523887. These sequences represented 5 phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria) consisting of 17 different genera. Proteobacteria was the most widely represented phylum with *Acetobacter, Burkholderia, Comamonas, Magnetospirillum, Pandoraea, Rhizobium* and *Sternotrophomona*s genera. For this reason, classes within Proteobacteria were taken into account to evaluate the data. Bacteroidetes followed with four affiliated genera, namely, *Bacteroides, Dyadobacter, Elisabethkingia* and *Flavobacterium. Faecalibacterium, Lactococcus* and *Paenibacillus* genera made up for the Firmicutes phylum (supplementary material Table S1). Results revealed a co-dominance of Betaprotoebacteria and Bacteroidetes in RB during all operation with some fluctuations. In RA and PRA the class Betaproteobacteria was generally persistent all along the operation. Additionally, Bacteroidetes were abundant in early-mid stages of the operation while Alphaproteobacteria class took over at mid-late operation.

4. Discussion

The consortia established in the reactors were well adapted to lower pH, as both reactors were controlled at pH 4.5, and to aerobic conditions, as the

reactors were aerated. Differences in bacterial populations between RA and RB were due to the presence of pelleted fungal biomass (i.e. in RA, contrarily to RB, Firmicutes were present until Day 9 and Proteobacteria α were predominant from Day 42 onwards). Differences in removal percentages cannot be directly linked to laccase production, as RA showed high removal values even when laccase activity was very low or not detected at all. This decoupling between laccase activity and PhACs removal can be explained by the diverse removal pathways of PhACs, some of which can be removed by mechanisms other than the laccase system –notably the cytochrome P450 in the case of fungal systems (Blánquez et al., 2004; Jelic et al., 2012; Marco-Urrea et al., 2010). Therefore, removal capacity will be discussed in terms of microbial community shifts.

Despite the differences, good removals are observed in the analgesics and anti-inflammatories family in both reactors. Several microorganisms are known capable of degrading some compounds of this group. In particular, acetaminophen and ibuprofen have been largely studied and can be degraded by bacteria as well as fungi (Langenhoff et al., 2013; Nguyen et al., 2014). The two compounds account for the high removal of this family in both reactors. RB removal did not reach 80-90% until microorganisms fully colonized the reactor, around Day 18, when glucose reached near zero values. RA removal increase during the first 14 days could only be partially explained by the growth of microorganisms other than *T. versicolor*. A second contribution could be the increase in concentration of ketoprofen in RA, hence reducing the overall removal. *T. versicolor* can degrade well over 80% of ketoprofen in the same matrix and at the same HRT when the compound is in spiked concentration

(Mir-Tutusaus et al., 2016). This fact demonstrated that the concentration increase in this non-spiked matrix is probably due to deconjugation of glucuronide conjugates of ketoprofen, and that ketoprofen conjugated compounds were in fact at higher concentrations. This was in agreement with Jelic et al. (2015), which stated that an 80% of the ketoprofen is excreted of the human body as a glucuronide-conjugated. In addition, the ability of *T. versicolor* to cleave conjugates of pharmaceutical compounds has been reported before in similar fungal systems (Badia-Fabregat et al., 2015; Cruz-Morató et al., 2013). The described ketoprofen concentration augment was not mirrored in RB, indicating that the microbial consortium did not reverse-transform conjugated compounds or that the community also removed such reverse-transformed products.

The case of diclofenac is of interest because it is widely used and not efficiently removed in conventional WWTP (Verlicchi et al., 2012). It was present only in HWW1 and completely removed during the fungal treatment, in accordance with Cruz-Morató et al. (2013). The uninoculated reactor also achieved a >90% removal; diclofenac degraders have been found in activated sludge with associated removal rates as low as 40% (Bouju et al., 2016). To our knowledge, this is the first time diclofenac has been reported to be completely removed by biostimulated wastewater-native microorganisms, although with an HRT of 3 days. This was achieved under non-steady conditions in terms of TSS, although diclofenac removal was indeed constant. The responsible candidates could be bacteria within the Proteobacteria (*Burkholderia*, *Comamonas* or *Microvirgula*) and Bacteroidetes (*Elisabethkingia*) phyla or fungi as *Asterotremella* or *Phialemoniopsis*. The genus *Elisabethkingia* contains species associated with

meningitis and to this date none of them have been described (nor studied) as degraders. Some species of *Burkholderia* have been found to degrade several pollutants such as chlorinated compounds (Zhang et al., 2013). *Comamonas* representatives have been found to degrade steroids and 4-chlorophenol (Linares et al., 2008; Tobajas et al., 2012). *Microvirgula* is a well-known genus of aerobic denitrifiers, which has also been reported to degrade several dyes (Han et al., 2012). Regarding the fungal candidates, *Phialemoniopsis* genus is usually related to eye infections and not reported to degrade PhACs. *Asterotremella* proliferation in the fungal pellets, as can be seen in Fig. 5, correlated with the decrease in PhACs removal. Microscopic observations were not carried out so this fact could not be confirmed. Although the liquid fraction in the pellet samples is very low, the *Asterotremella* percentage is higher than the corresponding to the liquid fraction, so it evidences an interaction between fungi. In addition, scarcely any references can be found about the yeast and none of them regarding its ability to biotransform any compounds. Thus, we propose that *Asterotremella* was not involved in the biotransformation of diclofenac.

Antibiotics are resistant to bacterial biodegradation but not to fungal degradation. This trend can be observed in Fig. 3 and Table 2, where no removal is appreciated in the uninoculated reactor. Contrarily, the fungal reactor, whose main biomass was pelleted fungi, showed very high removals of all the antibiotics detected during HWW1. The decrease in removal efficiency of RA could be clearly attributed to the loss of predominance of *T. versicolor* to *Asterotremella* in the fungal pellets, as seen in Fig. 5. The fungal reactor behaved equally as RB during HWW2 treatment, with no antibiotics removal. As

presence of *T. versicolor* is demonstrated by DGGE results (Fig. 5) and activity of the fungus, by laccase activity results (Fig. 1.), the absence of removal could be attributed to inhibition of *T. versicolor* or its degrading enzymes. *Pandoraea* and *Rhizobium* were the main bacterial genera present in the liquid matrix and pellets during the stated period. *Trichoderma* was present in the HWW and established without apparent difficulties in the non-inoculated bioreactor. However, antagonism with *Trametes* was expected to take place in reactor A to the detriment of *Trichoderma*, as *Trametes* was the one that prevailed. Similarly, *Isaria* was also abundant in the HWW but was not able to establish, not even in the control reactor. *Candida* was the main fungal genus in the liquid matrix and was present in the fungal pellets while no antibiotics removal was observed; additionally, a decrease in *Candida* between days 42-56 resulted in a slight increase in antibiotics removal. Therefore, *T. versicolor* inhibition could be caused by the presence of *Candida*.

The profile of psychiatric drugs concentration (excluding the carbamazepine family) in RA is very similar to the antibiotics profile in RA: a decrease in removal capacity is observed, well correlated with the invasion of the fungal pellets by *Asterotremella*. After the change in HWW, RA exhibited very low removal values, probably due to the hypotheses discussed above. Special attention can be paid to the antidepressant venlafaxine, a very recalcitrant compound typically detected in HWW and urban wastewaters (Evgenidou et al., 2015). Venlafaxine is usually poorly removed, even in similar fungal reactors in sterile conditions (Badia-Fabregat et al., 2015). RA removed up to 95% of venlafaxine at Day 7. Interestingly, similar results can be found in the bibliography using the same fungal system in non-sterile conditions.

Other pharmaceutical compounds included antihypertensives, anthelmintics, anticoagulants, β-blockers, diuretics, tamsulosin, H1 and H2 antagonists, lipid regulators and dexamethasone. The main contributors of this miscellanea family are gemfibrozil and ranitidine, with extremely different concentrations in HWW1 and HWW2. Gemfibrozil concentration was 6364 and 1921 $ng \cdot L^{-1}$ and ranitidine was 1830 and 20 $ng \cdot L^{-1}$ for HWW1 and HWW2 respectively. Both compounds could be removed well above 80% by RA and RB, except ranitidine in HWW2, where the initial concentration was very low. This combination of good degradability and lower initial concentrations between HWWs resulted in the decrease in overall removal of this miscellanea family observed in HWW2.

Carbamazepine removal in the fungal reactor was well correlated with *T. versicolor* presence in the fungal pellets in HWW1 and averaged 60%, which was in accord with the bibliography (Zhang and Geißen, 2012). The decreased removal of CBZ and 10,11-epoxyCBZ in HWW2 could be due to the already discussed *Candida* presence. The increase in acridone concentration was attributed to the biotransformation of CBZ, 10,11-epoxyCBZ and acridine, this last one not analyzed in this study (Golan-Rozen et al., 2015). The ability to completely remove 2-hydroxyCBZ remained unaltered during the whole treatment. Cruz-Morató et al. (2013) also found an increase in acridone concentration when non-sterile wastewater was treated, but complete removal when treating a sterile matrix. Contrarily, 46% removal of 2-hydroxyCBZ was achieved in sterile conditions while in our study the complete removal was obtained in non-sterile matrices. Therefore, other microorganisms may play a role in acridone accumulation and 2-hydroxyCBZ removal in non-sterile fungal operations. Jelic et al. (2011) deduced that 10,11-epoxyCBZ could appear by

deconjugation of glucuronides. Glucoronidases are a type of transferase enzyme present in white-rot fungi and used in the catabolism of organic pollutants. Transferases catalyze the formation of glucoside, glucuronide, xyloside, sulphate or methyl conjugates from several compounds, increasing its solubility and reducing its toxicity (Harms et al., 2011). In fact, conjugation of xenobiotics has been widely reported (Hundt et al., 2000; Ichinose et al., 1999). The deconjugation of ketoprofen in the fungal reactor –but not in the uninoculated reactor has been suggested above. Therefore, it is proposed that *Trametes versicolor* could enhance the deconjugation of such compounds.

In general, the global removals of both treatments RA and RB are similar due to ibuprofen and acetaminophen being the main contributors to the overall PhACs concentration and both being easily removed. Activated sludge in WWTP is also reported to remove several of these compounds. However, when highly recalcitrant xenobiotics are taken in account, like the compounds in antibiotic and psychiatric drugs families, the fungal treatment overpowered the uninoculated reactor. These families are not only very recalcitrant but also the main contributors to effluent overall toxicity and therefore environmental risk (Lucas et al., 2016). Fungal effluent exhibited lower concentrations of such products and lower toxicity values, as discussed below.

In an attempt to evaluate the capacity of both reactors to degrade or transform other compounds not included in the chemical analysis an acute toxicity bioassay with the bacterium *Vibrio fischeri* was performed. In addition, the approach could be used to evaluate the risk involved with the disposal of a potentially fungal-treated and non-treated hospital effluent into the sewage system. The Environmental Protection Agency (2004) recommends 0.3 TU as a

threshold for acute toxicity and 1.0 TU for chronic toxicity. The fungal treatment succeeded in maintaining the acute toxicity at 0.0 TU during the whole treatment. This indicated that *T. versicolor* removed non-analyzed toxic compounds and that no toxic metabolites were generated, or that potential toxic intermediates were also degraded, as pointed out by Cruz-Morató et al. (2013). The absence of toxicity suggested the possibility of disposal of the effluent to the sewage system. Contrarily, the uninoculated reactor raised the acute toxicity of the initial HWW to 27 TU, implying that the effluent should not be disposed of, that non-analyzed toxic compounds were not removed or that toxic metabolites were formed –and not degraded.

5. Conclusions

T. versicolor in pelleted morphology was maintained in a fungal reactor treating flocculated non-sterile real hospital wastewater for two months with an HRT of 3 d. A partial biomass renovation strategy was used to maintain *T. versicolor* activity throughout the treatment, with a CRT of 21 d. A DGGE and sequencing approach confirmed that *T. versicolor* survived during the whole treatment. Regardless, longer operations might be needed to achieve a steady community structure.

81 pharmaceutical compounds were analyzed and 46 were detected. Fungal treatment consistently removed most of the detected PhACs, including the most recalcitrant ones. Treated wastewater effluent did not exhibit any toxicity and therefore the operation might have removed potential toxic metabolites. Some interspecies interactions favored and some obstructed removal of some PhACs.

To the best of the authors' knowledge this is the first time a fungal treatment was implemented for 2 months treating non-sterile HWW.

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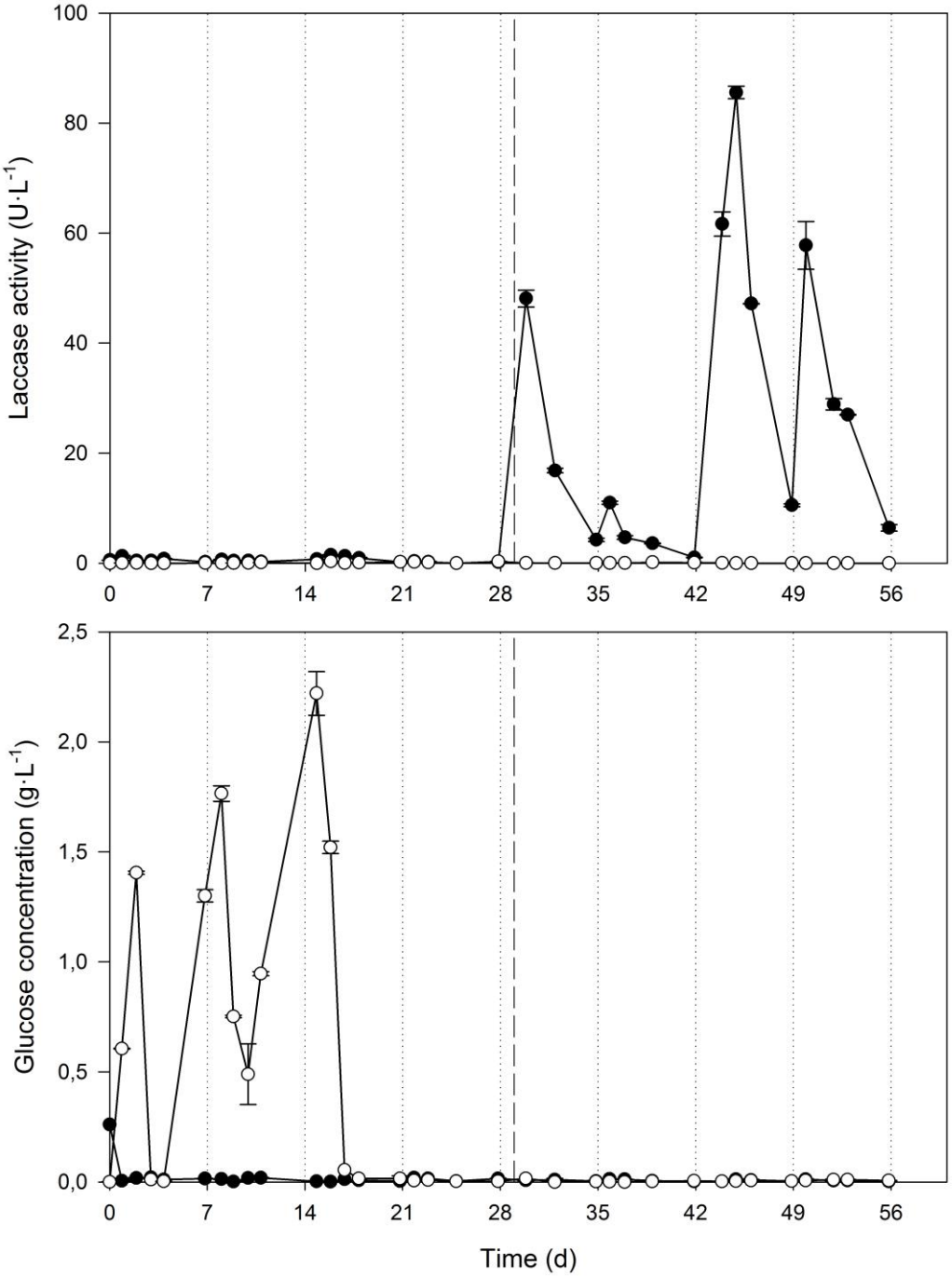

Fig.1. Evolution of laccase activity and glucose concentration during the continuous treatments with an HRT of 3 d. Black circles represent the reactor with *T. versicolor* and white circles, the uninoculated reactor. Vertical dotted lines represent weekly partial biomass renovation; the vertical dashed line, the change of wastewater.

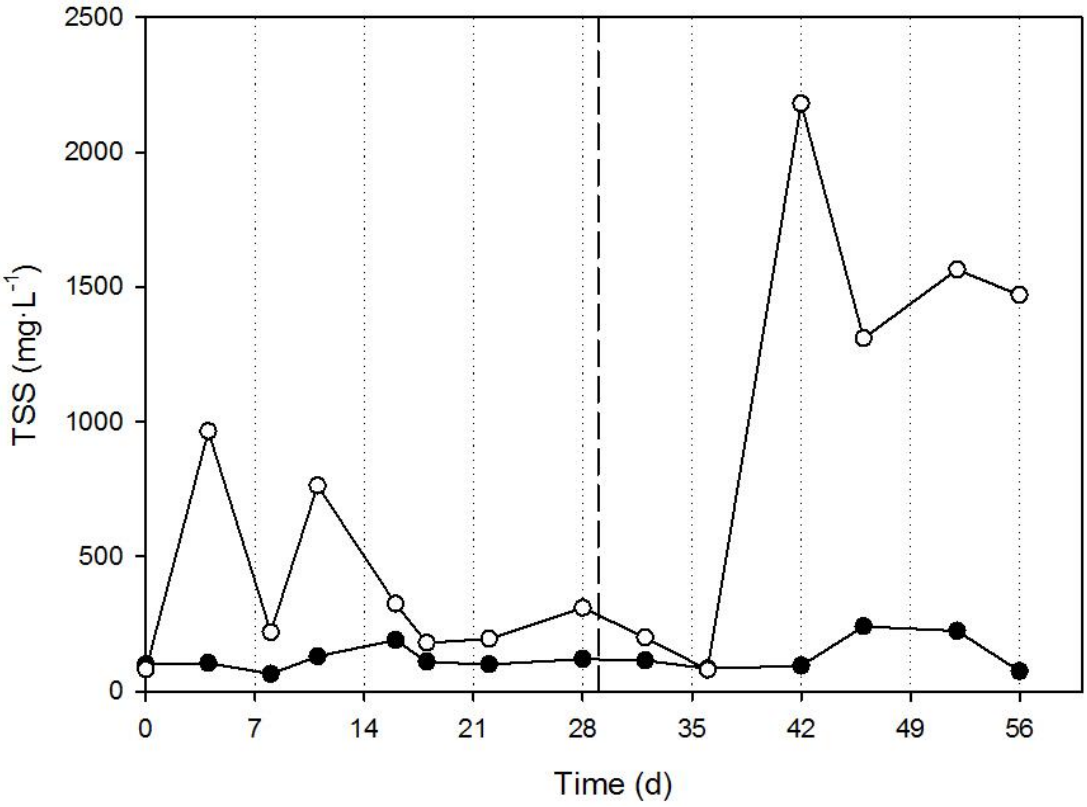

Fig.2. Evolution of total suspended solids concentration during the treatments. Black circles represent the reactor with *T. versicolor*; white circles, the uninoculated reactor. Dotted lines represent weekly partial biomass renovation; the dashed line, the change of wastewater.

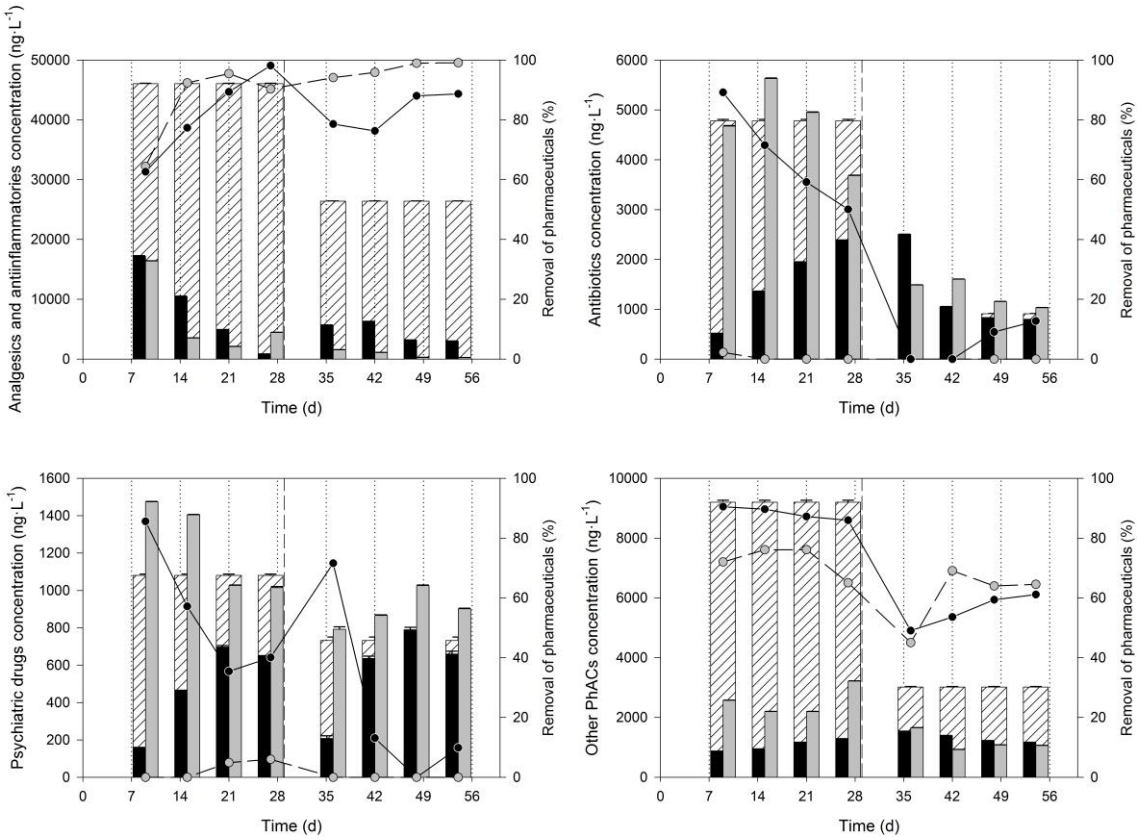

Fig.3. Pharmaceuticals concentration (in bars) and degradation (in circles) by families: analgesics and anti-inflammatories (top left), antibiotics (top right), psychiatric drugs not including carbamazepine and carbamazepine transformation products (bottom left) and the rest of PhACs (bottom right). Black bars/circles represent the reactor with *T. versicolor* and gray bars/circles, the uninoculated reactor; white patterned bars, the initial concentrations of each family. Dotted lines represent weekly partial biomass renovation; the dashed line, the change of wastewater.

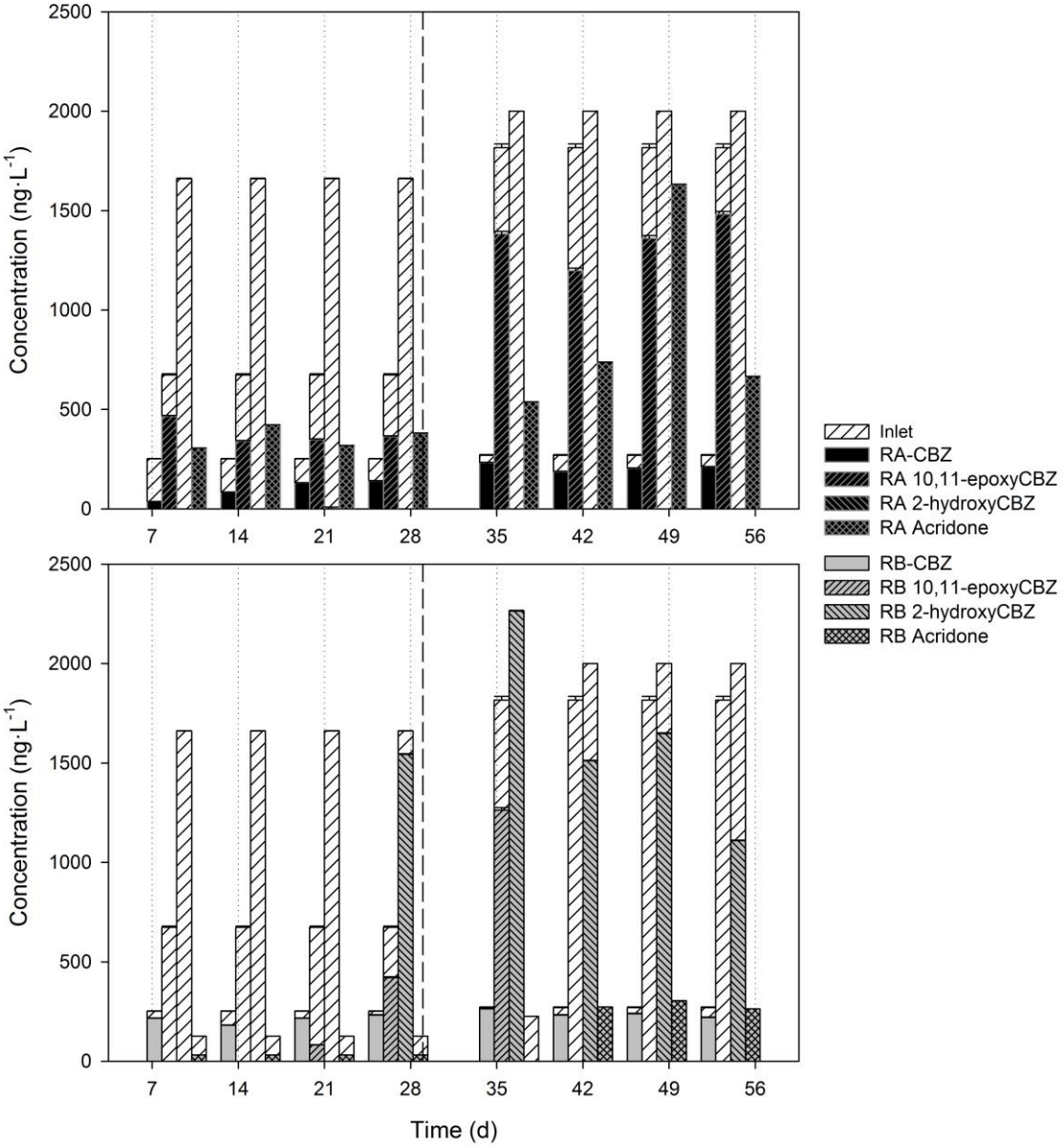

Fig.4. Carbamazepine and carbamazepine transformation products concentration. Black bars represent the reactor with *T. versicolor* and gray bars, the uninoculated reactor; white patterned bars, the initial concentration of each product. Dotted lines represent weekly partial biomass renovation; the dashed line, the change of wastewater.

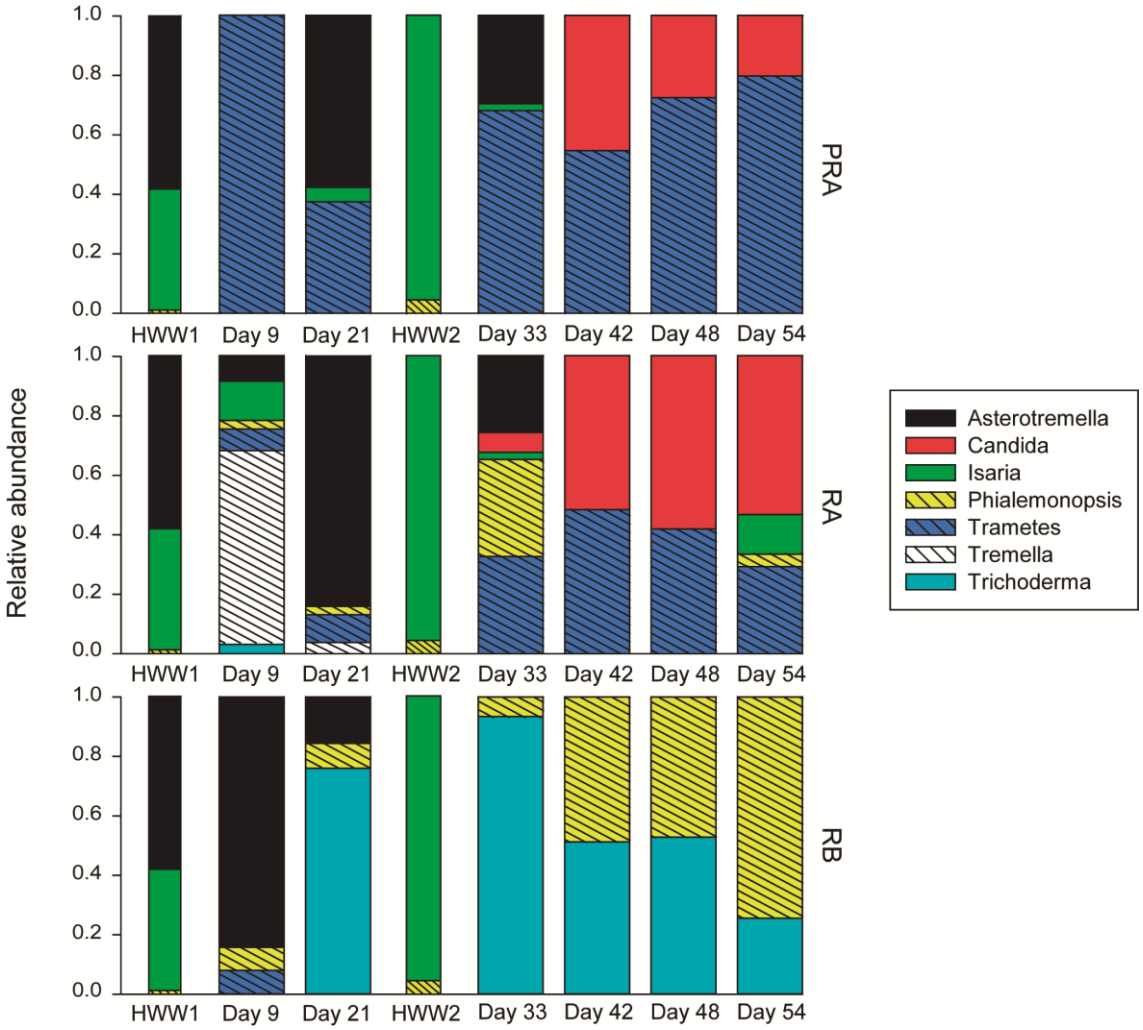

Fig.5. Phylogenetic assignment of fungal sequences from the HWWs, the liquid matrix (RA) and pelleted biomass (PRA) of the inoculated reactor, and from the liquid matrix of the non-inoculated reactor (RB). Data is presented in form of relative abundance, previously calculated with a semi-quantitative DGGE matrix and sequenced bands from the DGGE gels. Narrow bands represent the initial fungal composition of the two wastewaters; broad bands, the fungal composition during the treatments.

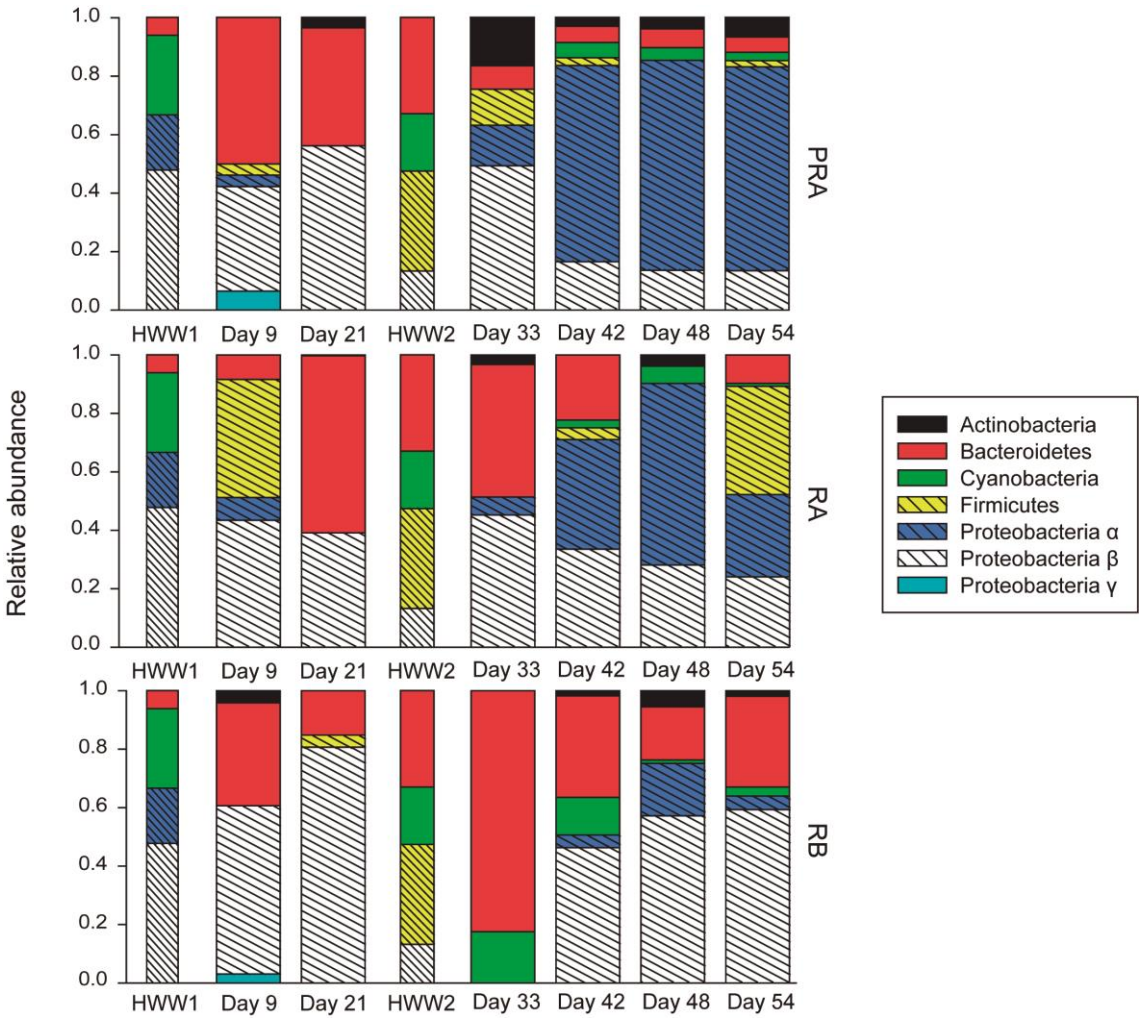

Fig.6. Phylogenetic assignment of bacterial sequences from the HWWs, the liquid matrix (RA) and pelleted biomass (PRA) of the inoculated reactor, and from the liquid matrix of the non-inoculated reactor (RB). Data is presented in form of relative abundance, previously calculated with a semi-quantitative DGGE matrix and sequenced bands from the DGGE gels. Narrow bands represent the initial bacterial composition of the two wastewaters; broad bands, the bacterial composition during the treatments.

Table 1.Physicochemical characterization of the hospital wastewaters.

Sampling date	14/09/2015		26/10/2015	
	HWW1		HWW2	
	Non flocculated	Flocculated	Non flocculated	Flocculated
pH	7.8	8.1	8.7	8.0
Conductivity ($mS \cdot cm^{-1}$)	1.99	1.98	1.69	1.29
Absorbance at 650 nm	0.215	0.000	0.265	0.012
Chloride ($mg\ Cl \cdot L^{-1}$)	284.3	315.0	271.3	216.5
Sulfate ($mg\ S \cdot L^{-1}$)	67.1	73.1	59.9	43.6
Nitrate ($mg\ N \cdot L^{-1}$)	0.0	0.1	4.1	2.7
Phosphate ($mg\ P \cdot L^{-1}$)	1.3	2.0	0.5	0.2
Ammonia ($mg\ N \cdot L^{-1}$)	36.0	35.6	9.9	9.7
TSS ($mg \cdot L^{-1}$)	284	58	193	93
COD ($mg\ O2 \cdot L^{-1}$)	633	215	1012	300
DIC ($mg \cdot L^{-1}$)	94 ± 1	71 ± 1	49 ± 1	39 ± 1
DOC ($mg \cdot L^{-1}$)	50 ± 4	53 ± 4	211 ± 11	56 ± 5

Table 2. Pharmaceuticals initial concentration and removal percentage during the treatments.

Therapeutical group	Compound	HWW1			HWW2		
			RA	RB		RA	RB
		C (ng·L-1)	Removal (%)	Removal (%)	C (ng·L-1)	Removal (%)	Removal (%)
Analgesics and anti-inflammatories	Acetaminophen	> 20000	> 99.3 ± 0	> 85.7 ± 28	> 20000	> 99.5 ± 1	100.0 ± 0
	Diclofenac	951.3	99.8 ± 0	95.9 ± 8	bld	-	-
	Ibuprofen	>20000	> 85.5 ± 19	> 88.7 ± 6	3960.14	84.6 ± 2[b]	93.0 ± 2[b]
	Ketoprofen	5109.3	-3.6 ± 84	71.5 ± 20	2432.33	-54.4 ± 72[b]	79.8 ± 25[b]
	Phenazone	bld	-	-	9.23	-314.0 ± 139	-147.3 ± 192
	Total	46061.9	81.9 ± 15	85.6 ± 14	26404.07	82.9 ± 6[b]	97.0 ± 2[b]
Anthelmintics	Thiabendazole	blq	70.0 ± 0	70.0 ± 0	9.10	97.7 ± 0[b]	92.3 ± 0[b]
	Albendazole	blq	-	-1338.7 ± 2724	blq	-	-
	Total	0.9	53.3 ± 0	-266.8 ± 651	9.32	95.4 ± 0[b]	90.1 ± 0[b]
Antibiotics	Azithromycin	bld	-	-	45.36	-95.5 ± 107[b]	88.5 ± 21[b]
	Ciprofloxacin	366.4	47.1 ± 25	-32.8 ± 67	266.90	-7.2 ± 16	-2.5 ± 29
	Ronidazole	bld	-7745.2 ± 15490	-16726.3 ± 3627	bld	-18135.5 ± 1690	-28321.6 ± 9351
	Sulfamethoxazole	1130.4	78.2 ± 9[a]	29.0 ± 31[a]	55.85	34.8 ± 76	-42.6 ± 87
	Trimethoprim	748.3	52.3 ± 35[a]	-27.8 ± 54[a]	81.80	-26.9 ± 96	-42.1 ± 67
	Ofloxacin	2537.1	71.1 ± 14[a]	3.8 ± 21[a]	459.39	-57.8 ± 168	-65.4 ± 54
	Total	4783.0	67.5 ± 17[a]	1.0 ± 17[a]	909.60	-42.3 ± 89	-45.0 ± 30
Anticoagulants	Warfarin	10.0	94.8 ± 0	94.8 ± 0	bld	-	-
	Total	10.0	94.8 ± 0	94.8 ± 0	bld	-	-
Antihypertensives	Valsartan	112.7	34.2 ± 22	43.4 ± 46	55.46	-44.6 ± 63	15.6 ± 18
	Total	112.7	34.2 ± 22	43.4 ± 46	55.46	-44.6 ± 63	15.6 ± 18
b-blockers	Atenolol	59.4	14.3 ± 37[a]	78.7 ± 23[a]	154.45	67.5 ± 24	60.9 ± 19
	Propanolol	bld	-	-	bld	blq	blq
	Sotalol	251.6	-151.9 ± 29	-205.2 ± 65	bld	-10442.4 ± 20885	-17997.6 ± 35995
	Total	327.5	-114.1 ± 27	-143.3 ± 54	171.16	32.9 ± 23	3.5 ± 41
Diuretics	Furosemide	bld	-664.4 ± 1431	-1018.9 ± 2124	161.88	97.6 ± 0	96.2 ± 3
	Hydrochlorothiazide	408.4	58.4 ± 34	23.1 ± 14	650.78	10.9 ± 12	21.8 ± 2
	Total	412.3	51.6 ± 45	13.2 ± 10	812.66	28.2 ± 10	36.6 ± 1
Drug against prostatic hyperplasia	Tamsulosin	bld	-	-58.3 ± 117	7.30	41.6 ± 5[b]	98.7 ± 0[b]
	Total	bld	-	-58.3 ± 117	7.30	41.6 ± 5[b]	98.7 ± 0[b]
Histamine H1 and H2 receptor antagonists	Ranitidine	1830.3	96.7 ± 3	89.9 ± 6	20.43	-2.4 ± 107	-77.3 ± 61
	Loratadine	1.2	46.9 ± 33	75.5 ± 0	bld	-89.6 ± 113	-116.7 ± 135
	Total	1831.5	96.7 ± 3	89.8 ± 6	20.71	-3.6 ± 105	-77.9 ± 62
Lipid regulators	Atorvastatin	14.9	94.9 ± 7	90.1 ± 15	13.77	95.6 ± 2	71.1 ± 28
	Fluvastatin	bld	-	-306.2 ± 612	bld	-	-
	Gemfibrozil	6364.1	100.0 ± 0[a]	83.6 ± 3[a]	1921.34	85.1 ± 19	90.3 ± 19
	Total	6381.8	99.9 ± 0[a]	83.5 ± 3[a]	1937.80	85.1 ± 19	90.1 ± 19
Psychiatric drugs	10.11-epoxyCBZ	673.2	43.6 ± 9	81.3 ± 30	1816.91	25.4 ± 6[b]	82.6 ± 35[b]
	2-hydroxyCBZ	1661.1	99.9 ± 0	76.7 ± 46	> 2000	> 74.9 ± 50[b]	> 18.3 ± 24[b]
	Acridone	126.0	-183.5 ± 43[a]	74.5 ± 0[a]	225.85	-295.8 ± 221[b]	6.5 ± 62[b]
	Alprazolam	bld	-	-	10.04	-17.7 ± 41	4.4 ± 41
	Carbamazepine	251.4	61.0 ± 19[a]	16.0 ± 8[a]	270.09	22.7 ± 7	11.6 ± 7
	Citalopram	297.4	39.0 ± 18[a]	-18.2 ± 5[a]	351.00	-4.4 ± 38	-22.0 ± 14
	Diazepam	bld	-58.3 ± 117	-	bld	-58.3 ± 117	1.9 ± 4
	Fluoxetine	bld	-	-	bld	-	-
	Norfluoxetine	12.5	90.1 ± 0	-26.5 ± 233	bld	-	-
	Olanzapine	131.5	98.7 ± 2	-44.6 ± 153	66.98	99.2 ± 0[b]	-73.9 ± 104[b]
	Setraline	98.8	98.3 ± 0	98.3 ± 0	127.85	98.7 ± 0	55.1 ± 50
	Razodone	36.3	98.0 ± 0[a]	-112.5 ± 94[a]	20.86	69.7 ± 54	29.7 ± 54
	Venlafaxine	495.3	41.0 ± 41[a]	-18.2 ± 7[a]	146.18	-19.2 ± 79	-78.8 ± 46
	Total	3791.9	54.8 ± 24	47.6 ± 22	5044.96	30.0 ± 24	34.7 ± 18
Synthetic glucocorticoid	Dexamethasone	121.8	77.2 ± 44	33.5 ± 76	bld	-34501.3 ± 23481	-32983.9 ± 23315
	Total	121.8	77.2 ± 44	33.5 ± 76	bld	-34501.3 ± 23481	-32983.9 ± 23315

Bql: below limit of quantification; bld: below limit of detection. [a,b] Statistically different ($p < 0.05$).

Table 3. Chemical oxygen demand of effluent of reactors during the treatment.

Time (d)	COD ($mg \cdot L^{-1}$)	
	RA	RB
0	215	215
7	358	1900
21	3141	1115
42	379	1260
56	292	1335

Supporting Information: Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation-flocculation pretreatment

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Supporting information: 1 figure, 1 table. 3 pages in total.

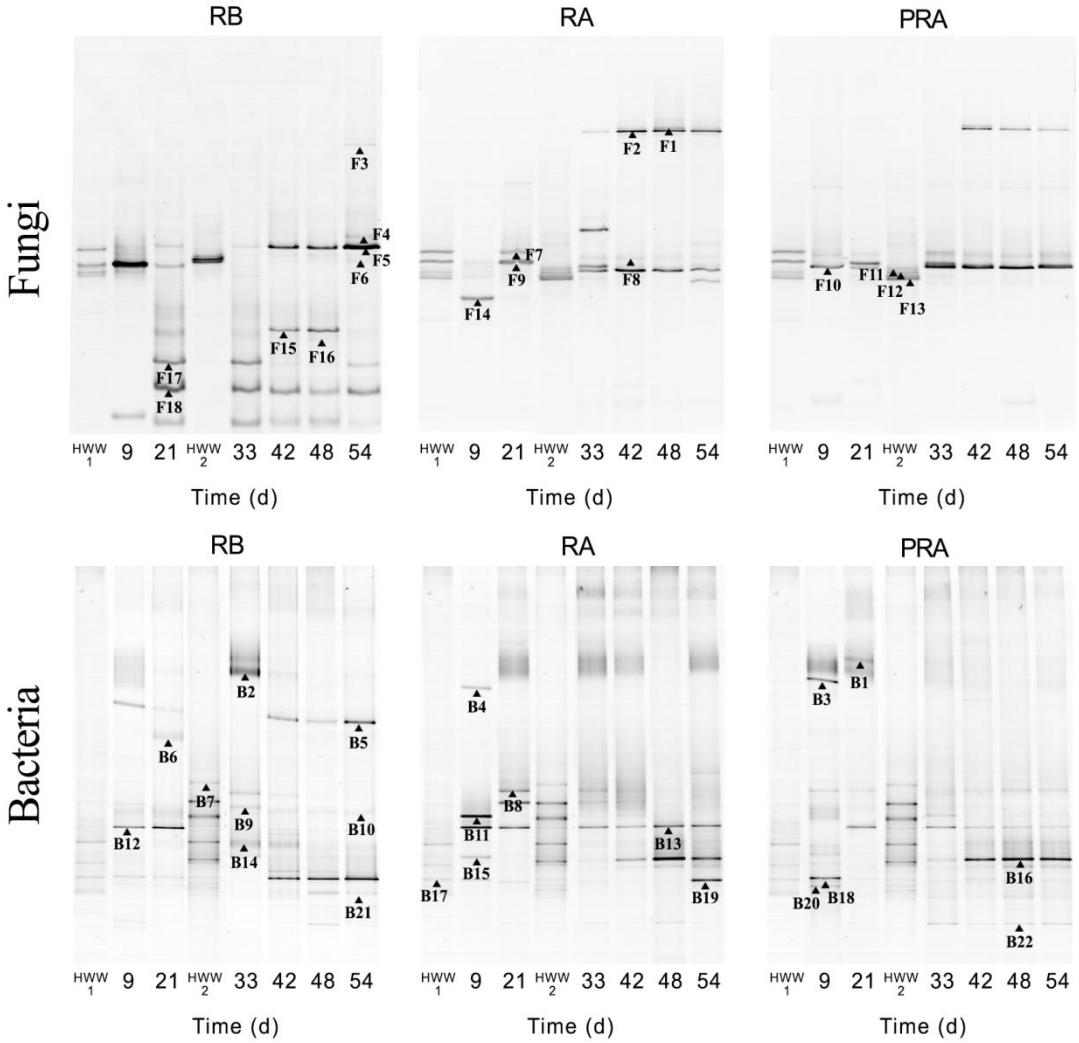

Fig. S1. DGGE profiles of fungal and bacterial communities detected in the inoculated reactor (RA), pellet fraction of the inoculated reactor (PRA) and non-inoculated reactor (RB) using the primer sets EF4-ITS4/ITS1-ITS2 and 341f-907r respectively. Representative bands (▲) are labeled according to their source. (F) Fungi, (B) Bacteria.

Table S1. Phylogenetic affiliations of bacterial 16S rRNA gene and fungal ITS sequences obtained from the reactors after DGGE.

DGGE band	Closest cultured BLAST match	Accession number	Similarity (%)	Phylogenetic affiliation (Phylum)
F01-02	*Candida sojae*	KJ722419	100	Ascomycota
F03-06	*Phialemoniopsis curvata*	AB278180	98	Ascomycota
F07, F9	*Asterotremella humicola*	KC118118	100	Basidiomycota
F08, F10	*Trametes versicolor*	KR261581	100	Basidiomycota
F11-13	*Isaria* cf. *farinosa*	FN548150	99	Ascomycota
F14	*Tremella exigua*	KP986514	100	Basidiomycota
F15	*Trichoderma asperellum*	KR856224	100	Ascomycota
F16-18	*Trichoderma asperellum*	KR856224	100	Ascomycota
B01	*Flavobacterium oncorhynchi*	KT354259	100	Bacteroidetes
B02	*Flavobacterium* sp.	JF915323	99	Bacteroidetes
B03-04	*Elizabethkingia miricola*	LN995715	100	Bacteroidetes
B05	*Chryseobacterium meningosepticum*	AF207076	100	Bacteroidetes
B06	*Bacteroides oleiciplenus*	NR_113070	95	Bacteroidetes
B07	*Faecalibacterium prausnitzii*	HQ457025	100	Firmicutes
B08	*Dyadobacter fermentans*	LN890052	100	Bacteroidetes
B09	*Dyadobacter* sp.	DQ207362	100	Bacteroidetes
B10	*Microvirgula aerodenitrificans*	LN997979	100	Proteobacteria (β)
B11	*Lactococcus lactis*	KU942499	100	Firmicutes
B12	*Burkholderia gladioli*	KT862889	100	Proteobacteria (β)
B13	*Pandoraea sputorum*	LN995687	100	Proteobacteria (β)
B14	Cyanobacterium TDX16	KJ599678	95	Cyanobacteria
B15	*Acetobacter aceti*	KR261398	99	Proteobacteria (α)
B16	*Rhizobium* sp.	KT387839	100	Proteobacteria (α)
B17	*Comamonas aquatica*	LN558648	99	Proteobacteria (β)
B18	*Comamonas aquatica*	KT716080	99	Proteobacteria (β)
B19	*Paenibacillus* sp.	JX469414	99	Firmicutes
B20	*Stenotrophomonas* sp.	KR922087	100	Proteobacteria (γ)
B21	*Magnetospirillum* sp.	KM289194	99	Proteobacteria (α)
B22	Microbacteriaceae bacterium	KR082269	100	Actinobacteria

Abbreviation: BLAST, Basic Local Alignment Search Tool.